Oxytocin stimulates secretory processes in lactating rabbit mammary epithelial cells

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Oxytocin plays a major role in lactation mainly by its action on milk ejection via the contraction of myoepithelial cells. The effect of oxytocin on milk production and the presence of oxytocin receptors on different epithelial cells suggest that this hormone may play a role in mammary epithelial cells. To determine precisely the various roles of oxytocin, we studied localization of oxytocin receptors in lactating rabbit and rat mammary tissue and the influence of oxytocin on secretory processes in lactating rabbit mammary epithelial cells. Immunolocalization of oxytocin receptors on mammary epithelial cells by immunofluorescence and in mammary tissue by immunogold in addition to *in situ* hybridization showed that lactating rat and rabbit mammary epithelial cells expressed oxytocin receptors. Moreover, oxytocin bound specifically to epithelial cells. To determine whether oxytocin had an effect on lactating rabbit mammary epithelial cells, isolated mammary fragments were incubated in the presence or absence of 10^{-6} i.u. ml^{-1} of oxytocin. After 1 min of incubation with oxytocin, the morphology of epithelial cells and the localization of caseins and proteins associated with the secretory traffic suggested a striking acceleration of the transport leading to exocytosis, whereas the contraction of myoepithelial cells was only detectable after 7 min. Addition of 10^{-8} g ml^{-1} of atosiban before the addition of oxytocin prevented the oxytocin effect on secretory processes and on myoepithelial cell contraction. Addition of 10^{-6} i.u. ml^{-1} of vasopressin to the incubation medium did not mimic the stimulating effect of oxytocin on secretory traffic. These results show that lactating rabbit and rat mammary epithelial cells express oxytocin receptors and that oxytocin binds to these receptors. They strongly suggest that oxytocin has a dual effect on lactating mammary tissue: an acceleration of the intracellular transfer of caseins in mammary epithelial cells followed by the contraction of myoepithelial cells.

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Oxytocin is a neurohormone produced in the hypothalamo-neurohypophysial system, released in the systemic circulation by specific stimuli and transported to the target tissues. Oxytocin exerts multiple central and peripheral functions, including milk ejection, uterine smooth muscle contraction during labour, control of sexual and social behaviours, regulation of natriuresis and control of cardiovascular homeostasis (for a review, see Gimpl & Fahrenholz, 2001). One of the major physiological functions of oxytocin is to provoke milk ejection from the lactating mammary gland by eliciting contraction of the myoepithelial cells which surround the alveoli and the small intralobular ductules. This contraction causes collapse of the alveolar lumen and milk transfer through the ductules towards the cistern (in ruminants) and teats for eventual milk removal (Ely & Petersen, 1941). This role is essential in rodents since mice deficient in oxytocin after invalidation of the oxytocin gene are unable to nurse their offspring (Nishimori *et al.* 1996; Wagner *et al.* 1997).

Oxytocin effects are mediated by one type of oxytocin receptor encoded by only one oxytocin receptor gene, which has been cloned and sequenced in humans (Kimura *et al.* 1992), cows (Bathgate *et al.* 1995), ewes (Riley *et al.* 1995), rats (Rozen *et al.* 1995) and mice (Kubota *et al.* 1996). The oxytocin receptor belongs to the trimeric G-coupled protein receptor family (Burbach *et al.* 1995). The oxytocin receptor is expressed in various

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tissues, including pituitary, ovary, endometrium and myometrium, but also kidney, heart, vascular endothelium and mammary gland (for a review, see Gimpl & Fahrenholz, 2001). In the lactating rat mammary gland, oxytocin binding to specific sites (Soloff *et al.* 1980) and oxytocin receptor immunolocalization in myoepithelial cells (Adan *et al.* 1995) have been reported. These localizations are in line with its role on smooth muscle contraction. However, the presence of oxytocin receptors has also been observed in mammary epithelial cells from non-lactating and lactating humans and marmosets (Kimura *et al.* 1998) and in human breast cancer cells, which are typically of epithelial origin (Cassoni *et al.* 1994; Ito *et al.* 1995; Bussolati *et al.* 1996; Copland *et al.* 1999).

Moreover, it has been reported that oxytocin injections during lactation increase milk yield (Nostrand *et al.* 1991; Ballou *et al.* 1993; Knight, 1994). This increase of milk production could be explained by more efficient milk ejection due to the effect of oxytocin on myoepithelial contraction, but also to other effects. The presence of oxytocin receptors in mammary epithelial cells suggests that this hormone plays a role in this cell type. A previous study has shown that addition of oxytocin *in vitro* to lactating rabbit mammary fragments stimulated the intracellular transit of caseins in mammary epithelial cells and increased their secretion, by stimulating the intracellular transport of newly synthesized proteins between the rough endoplasmic reticulum (RER), the Golgi apparatus and the secretory vesicles (Ollivier-Bousquet, 1976). All these results have led to a re-examination of the role of oxytocin on different mammary tissue cell types.

The aims of the present work were to precisely localize oxytocin receptors in lactating rabbit and rat mammary tissue and to elucidate one of the potential roles of oxytocin in lactating rabbit mammary epithelial cells, with special emphasis on its effects on the intracellular process of milk secretion.

Methods

Animals

New Zealand White female rabbits and Wistar female rats on day 15 of lactation originated from our laboratory. Rabbits and rats were killed by cervical dislocation and by decapitation, respectively, and their mammary glands were excised. The ethical aspects of animal care complied with the relevant guidelines and licensing requirements laid down by the Ministère de l'Agriculture, France.

Materials

Hanks' medium (Hanks' Balanced Salt Solution, 10X) was purchased from Gibco (BRL-Life Technologies, Cergy-Pontoise, France), oxytocin (Syntocinon) from Novartis Pharma (Rueil-Malmaison, France), arginine-vasopressin from Sigma (St Louis, USA), Fluo-Oxytocin and the tyramide signal amplification (TSA) system from NEN Life Science (Boston, MA, USA). Atosiban was a generous gift of Ferring Research Institute (Malmö, Sweden). Monoclonal antibodies against human oxytocin receptor (2F8) were a generous gift from Dr T. Kimura (Ito *et al.* 1996) (see Acknowledgements). Polyclonal antibodies against oxytocin receptor (OTRec) were a generous gift from Dr F. Van Leeuwen (Adan *et al.* 1995). Polyclonal antibodies against α_{s1} casein were a generous gift from Dr L. M. Houdebine. Fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG was obtained from Jackson ImmunoResearch Laboratory (West Grove, PA, USA). Polyclonal antibodies against annexin II were purchased from Santa Cruz Laboratory (Santa Cruz, CA, USA). Partial rat oxytocin receptor cDNA subcloned into pGEM-7Zf+ was a generous gift from Professor J. P. H. Burbach and Dr D. Guénot. All reagents for *in situ* hybridization were purchased from Roche (Meylan, France). Unicryl for immunoelectron microscopy was purchased from Tebu (Le Perray en Yvelines, France) and the goat anti-mouse antibodies conjugated to gold particles were from Biocell (Cardiff, UK). All other reagents were obtained from Sigma.

Preparation and incubation of mammary gland fragments and acini

Each experiment was carried out with mammary fragments from one animal. Assays were repeated with at least three different animals except for semi-quantitative evaluation of the effects of vasopressin and atosiban measured on micrographs, as specified in the text. Mammary tissues, dissected free of connective and adipose tissues and cut into small fragments, were incubated in Hanks' medium at pH 7.4, 37°C, atmosphere 95% O_2–5% CO_2. This experimental process allowed us to obtain functional mammary fragments (Lkhider *et al.* 2001). After washing, fragments were incubated for 1 and 7 min in the absence or presence of 10^{-6} i.u. ml^{-1} oxytocin or 10^{-6} i.u. ml^{-1} vasopressin, in the same medium. Some fragments were incubated in the presence of 10^{-8} to 4×10^{-8} g ml^{-1} of atosiban (an oxytocin receptor-blocking agent) for 3 min and then in the presence of 10^{-6} i.u. ml^{-1} of oxytocin for 1 and 7 min (See Supplemental material Figs S1–S3). Periods of 1 and 7 min were selected, because 1 min represented a short period after the addition of oxytocin and because the most apparent changes were observed after 7 min in *in vitro* conditions.

Different concentrations of oxytocin (from 10^{-3} to 10^{-6} i.u. ml^{-1}) have been tested. The concentration of 10^{-6} i.u. ml^{-1} (2.2 pg ml^{-1} or 2.2 fmol ml^{-1}), which was

able to induce effects on the mammary fragments without morphological damage, was used in the present *in vitro* studies. This concentration is lower than those measured *in vivo* in lactating rabbits since it has been reported at levels varying between 31 and 650 pg ml^{-1} in response to suckling in mid-lactation (Bisset *et al.* 1970; Fuchs *et al.* 1984) and consequently may be considered as physiologically relevant.

To prepare enzymatically dissociated acini, mammary fragments were incubated for 90 min at 37°C in Hanks' medium containing 200 i.u. ml^{-1} collagenase IV and 200 i.u. ml^{-1} hyaluronidase III, under an atmosphere of 95% O_2–5% CO_2, centrifuged, washed and then filtered through a strainer. Isolated cells were separated from the acini by three successive decantations for 15 min at 20°C in Hanks' medium. We have previously verified that in these experimental conditions, acini express α_{s1} caseins and are able to have an oxytocin-induced secretory activity.

Immunofluorescence

After incubation, mammary fragments were fixed in 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, infiltrated with 40% sucrose in phosphate-buffered saline (PBS) (4 h), frozen in liquid nitrogen and sectioned in 2 μm thick sections at −35°C with a Reichert Cryocut (Leica, Rueil-Malmaison, France). Sections were collected on poly L-lysine-coated glass slides.

Sections were sequentially incubated in 50 mM NH_4Cl–PBS (45 min), in PBS–1% bovine serum albumin (BSA) (45 min), in serum corresponding to the species in which secondary antibody was raised (1 : 4) in PBS–1% BSA (1 h) and in primary antibodies all diluted in PBS–1% BSA (2 h 30 min) (anti-rabbit α_{s1} casein (1 : 1000), anti-p58 (1 : 60), anti-annexin II (1 : 20), anti-smooth muscle actin (1 : 5000), washed and then incubated with appropriate secondary antibody and tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin, 1 : 500 (30 min). Control sections were treated similarly with omission of the primary antibodies.

Acini were cytocentrifuged on poly L-lysine-coated glass slides and fixed in 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (1 h).

Fixed acini were permeabilized with Triton X-100 (0.1% in PBS, 1 min) and stained with Fluo-Oxytocin (50 nM). Staining was detected using the Tyramide Signal Amplification method, according to the instructions supplied by NEN. Control acini were incubated with 10^{-6} i.u. ml^{-1} of oxytocin before labelling with Fluo-Oxytocin (50 nM).

An anti-human oxytocin receptor monoclonal antibody named 2F8 (10 μg ml^{-1}) and a polyclonal antibody against oxytocin receptors named OTRec (1 : 500) were used for immunodetection of oxytocin receptors. Immunofluorescence was revealed with anti-mouse IgM-FITC (1 : 200) and anti-rabbit IgG-FITC (1 : 400), respectively.

Electron microscopy and immunoelectron microscopy

After the different treatments, fragments were fixed for 1 h in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C, postfixed in 1% OsO_4 in the same buffer, dehydrated in increasing-concentration acetone solutions and embedded in Epon 812. Polymerization was performed for 12 h at 60°C. Ultrathin sections were prepared and contrasted with uranyl and lead citrate.

Quantification of the effect of the different treatments (oxytocin, vasopressin and atosiban) was performed on electron micrographs of the apical region of the mammary epithelial cells taken at random. For each animal and each treatment, 10 micrographs were taken at a magnification of 3800 or 8000 and printed at a final magnification of 8400 and 18 000, respectively. The length of the apical membrane was measured. The number of secretory vesicles in close contact with the apical membrane and the number of vesicles located in the region of 2.5 μm closest to the apical membrane were counted. The number of vesicles per 100 μm of apical membrane was expressed as percentage. Quantification of the oxytocin effect was performed from three experiments corresponding to three animals. Quantification of the effects of vasopressin and atosiban was realized from tissues originating from one rabbit.

For oxytocin treatment, values from control and treated fragments carried out with fragments from a single rabbit were paired. The resulting pairs of values from three rabbits were compared using Student's *t* test.

For immunoelectron microscopy (immunoEM), mammary fragments were fixed in 4% paraformaldehyde containing 0.05% glutaraldehyde for 3 h at 4°C. Tissues were washed in PBS–0.4 M sucrose at 4°C overnight, dehydrated for 30 min each in ethanol at 30%, 50%, 70% and 90% at 4°C. They were then immersed at 0°C for 1 h each in two baths of Unicryl–ethanol 90% (1 vol. : 2 vol., 2 vol. : 1 vol., respectively), followed by two baths in Unicryl. Polymerization was performed at 320 nm UV for 24 h at 4°C and 72 h at room temperature. Ultrathin sections were mounted on Formwar-coated nickel grids.

Sections were incubated with 2F8 (the anti-human oxytocin receptor monoclonal antibody) (1 : 100, 1 : 250 or 1 : 1000) in TBS+ (20 mM Tris, pH 7.6, 150 mM NaCl, 0.1% ovalbumin, 0.1% Tween 20, 1% goat serum) for 60 min and washed in the same buffer. Antigen–antibody complexes were revealed by incubation for 1 h with a goat anti-mouse antibody (5×10^{-3} i.u. ml^{-1}) conjugated to 10 nm gold particles diluted 1 : 50 in TBS+ (pH 8.2). Grids were washed again, fixed in 2.5% glutaraldehyde in 2X saline sodium citrate (SSC) buffer, washed in 2X

SSC and then in distilled water, counterstained in 5% aqueous uranyl acetate (15 min), and air dried. Controls were carried out without the primary antibody. No major differences in oxytocin receptor intracellular localization were observed according to different 2F8 concentrations.

The distribution of gold particles were counted on five micrographs for each rabbit, taken at random at the same magnification of 6300. The gold particles on the different intracellular compartments (endoplasmic reticulum, apical membrane, basal membrane, nucleus, vesicles, mitochondria) were counted. The distribution of the gold particles in each compartment was expressed as a percentage of the total gold particles counted.

In situ hybridization

Partial rat oxytocin receptor cDNA was inserted into pGEM-7Zf+ and used to derive sense and antisense riboprobes. RNA probes were generated using digoxigenin-UTP, and either SP6 or T7 polymerases. The pGEM-7Zf+ construct was linearized with *Eco*R1 and transcript with SP6 RNA polymerase to obtain an antisense probe. Alternatively, the pGEM-7Zf+ construct was linearized with *Bam*H 1 and transcribed with T7 RNA polymerase to obtain a sense probe.

Lactating rat mammary glands were rapidly dissected out, frozen in liquid nitrogen and stored at −80°C until sectioning. Tissue sections (5 μm) were collected on glass slides (positively charged, Superfrost, VWR International, Fontenay sous Bois, France) fixed in 4% paraformaldehyde in PBS. After washing in PBS, sections were permeabilized with proteinase K (1 μg ml^{-1}) for 15 min at 37°C, then postfixed in 4% paraformaldehyde in PBS for 5 min, rinsed, dehydrated by graded concentrations of ethanol. Dry sections were covered with 20 μl of hybridization buffer containing the RNA probe (0.2 ng ml^{-1}) in 4-fold SSC (0.06 M sodium citrate, 0.6 M NaCl) buffer, 50% formamide, 1% Denhardt's solution (0.05% polyvinylpyrrolidone 40, 0.05% Ficoll 400, 0.02% BSA) and 250 μg ml^{-1} of yeast tRNA. After overnight incubation at 56°C in a humid chamber, slides were washed once in SSC buffer, twice at 50°C in 0.25-fold SSC buffer, once in 2-fold SSC buffer containing 10 μg ml^{-1} of RNase A, and twice in 2-fold SSC buffer.

For immunodetection, sections were first washed in Tris-HCl 50 mM, NaCl 300 mM, pH 7.6 and blocked in the same buffer containing 1% goat serum. Hybridized digoxigenin probes were reacted overnight with Fab fragments anti-digoxigenin (1 : 1000), conjugated to alkaline phosphatase. Finally, sections were washed twice in 50 mM Tris, 300 mM NaCl, pH 7.6 and 100 mM Tris, 300 mM NaCl, 50 mM $MgCl_2$, pH 9.5, and revealed in Nitro Blue Tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (NBT-BCIP). Purple staining was examined using a Zeiss DMBR microscope.

Results

Oxytocin receptors are detectable by immunofluorescence in rabbit and rat mammary epithelial cells

During lactation, mammary acini consist of mammary epithelial cells and myoepithelial cells which surround acini and small ducts. It is well documented that oxytocin receptors are associated with myoepithelial cells (Soloff *et al.* 1980; Adan *et al.* 1995). To detect whether oxytocin receptors were also associated with mammary epithelial cells, enzymatically dissociated acini enriched in epithelial cells were prepared. After permeabilization, immunofluorescence localization of oxytocin receptors was carried out on this preparation using the anti-human oxytocin receptor monoclonal antibody 2F8. Moreover, enzymatically dissociated acini were labelled with TRITC-phalloidin, which stains actin.

No labelling was detectable with the second antibody alone (Fig. 1*A*). Oxytocin receptor immunoreactivity was located in the epithelial cells that constitute the acini (Fig. 1*B*). Myoepithelial cells containing muscular actin filling the cytoplasm were strongly labelled by TRITC-phalloidin, whereas mammary epithelial cells surrounded by a thin layer of actin located under the plasma membrane were not strongly labelled. This made it possible to discriminate between myoepithelial cells and mammary epithelial cells and revealed that few myoepithelial cells remained associated with acini (Fig. 1*C* and *E*). Double localization of oxytocin receptor and actin showed that oxytocin receptors were detectable on the periphery and in the cytoplasm of the mammary epithelial cells surrounded by a thin layer of actin (Fig. 1*D* and *F*). The same localization was obtained with the polyclonal antibody OTRec (not shown).

Oxytocin receptors can be localized precisely in rabbit and rat mammary gland by immunogold

For the precise localization of oxytocin receptors in lactating mammary tissue, immunoEM localization was performed in lactating rabbit and rat mammary tissue with the anti-human oxytocin receptor monoclonal antibody 2F8.

Oxytocin receptors were detectable in myoepithelial, epithelial and endothelial cells.

In myoepithelial cells, the receptor was detectable on the plasma membrane and in very small and electron-dense structures located in the cytoplasm filled by numerous fibres of actin (Fig. 2*A*). No gold particles were detectable

in tissue sections treated with the second antibody alone (Fig. 2*B*). In epithelial cells, the receptor was detectable on the basal plasma membrane (Fig. 2*A* and *C*). In the cytoplasm of mammary epithelial cells, gold particles were associated with different types of intracellular vesicles, either coated vesicles close to the basal membrane or small and electron-dense structures (Fig. 2*C*). Oxytocin receptors were also detectable on mammary epithelial cell RER (Fig. 2*D*) and in small vesicles associated with the Golgi apparatus (Fig. 2*E*), but very rarely in secretory vesicles containing casein micelles. A semi-quantitative evaluation of the distribution of gold particles in the epithelial cells showed that 10 min after suckling 31% of the gold particles were present on the RER and 24% were present on or close to the basal membrane, whereas 24 h after suckling 24% were present on the RER and 21% on the basal membrane, which is the typical location for binding oxytocin during the next suckling (not shown).

Oxytocin receptors were very frequently located on the membrane and in vesicles of the capillary endothelial cells (Fig. 2*C*).

ImmunoEM localization of oxytocin receptors was the same in lactating rat mammary tissue (not shown).

Oxytocin receptor messenger RNAs are detectable in lactating rat mammary gland by *in situ* hybridization

Because oxytocin receptors are present in mammary epithelial cell RER, the next question to determine was whether these receptors are synthesized in this cell type. Evaluation of messenger RNAs (mRNAs) by RT-PCR would necessitate a fully purified preparation of mammary epithelial cells. As shown in Fig. 1, the acini preparation used in this work was strongly enriched in mammary epithelial cells but very few myoepithelial cells were present. For this reason, oxytocin receptor gene expression in lactating rat mammary gland was analysed by *in situ* hybridization, with rat mammary tissue slides, using a rat oxytocin receptor RNA probe. As shown in Fig. 3*B*, a specific hybridization signal was found in the cytoplasm of cells containing lipid globules. Because only epithelial cells contain lipid globules, it can be concluded that oxytocin receptor mRNAs are present in mammary epithelial cells. No labelling was observed when a corresponding sense RNA was used as a probe (Fig. 3*A*).

Fluo-Oxytocin binds to rabbit acini

Since oxytocin receptors were present in mammary epithelial cells, the next step was to test the ability of oxytocin to bind to mammary epithelial cells. To this aim, rabbit acini were stained with Fluo-Oxytocin. Fluo-Oxytocin was detectable as numerous fluorescent spots in the cytoplasm and colocalized with actin on the periphery of the mammary epithelial cells (Fig. 4*B* and *D*). Preincubation of acini with oxytocin impaired the binding of Fluo-Oxytocin (Fig. 4*A*), confirming that Fluo-Oxytocin binds to specific binding sites on the mammary epithelial cells. These results showed that mammary epithelial cells express specific binding sites able to bind oxytocin.

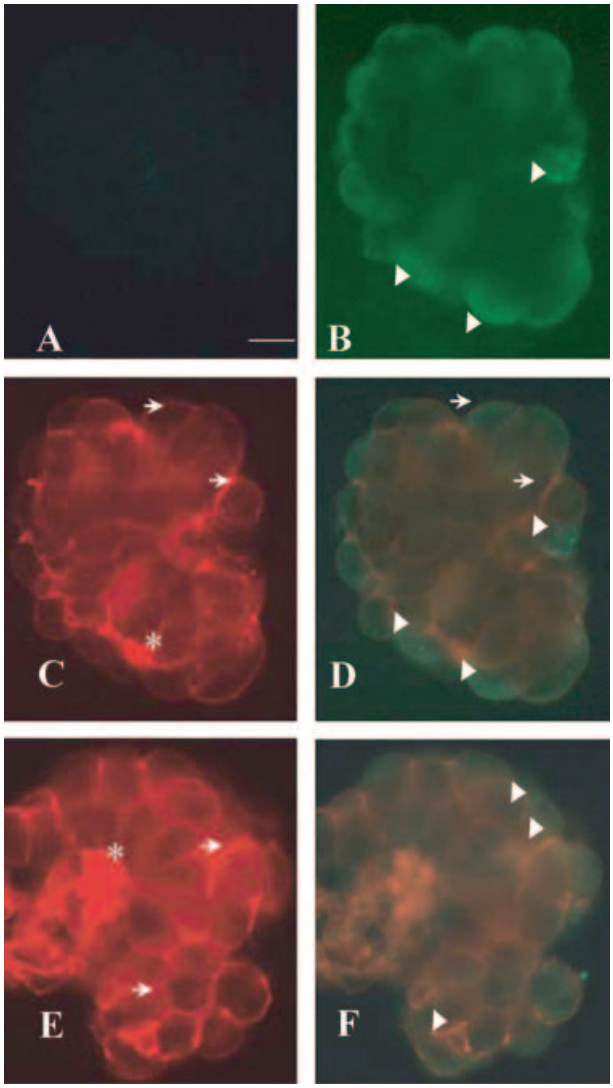
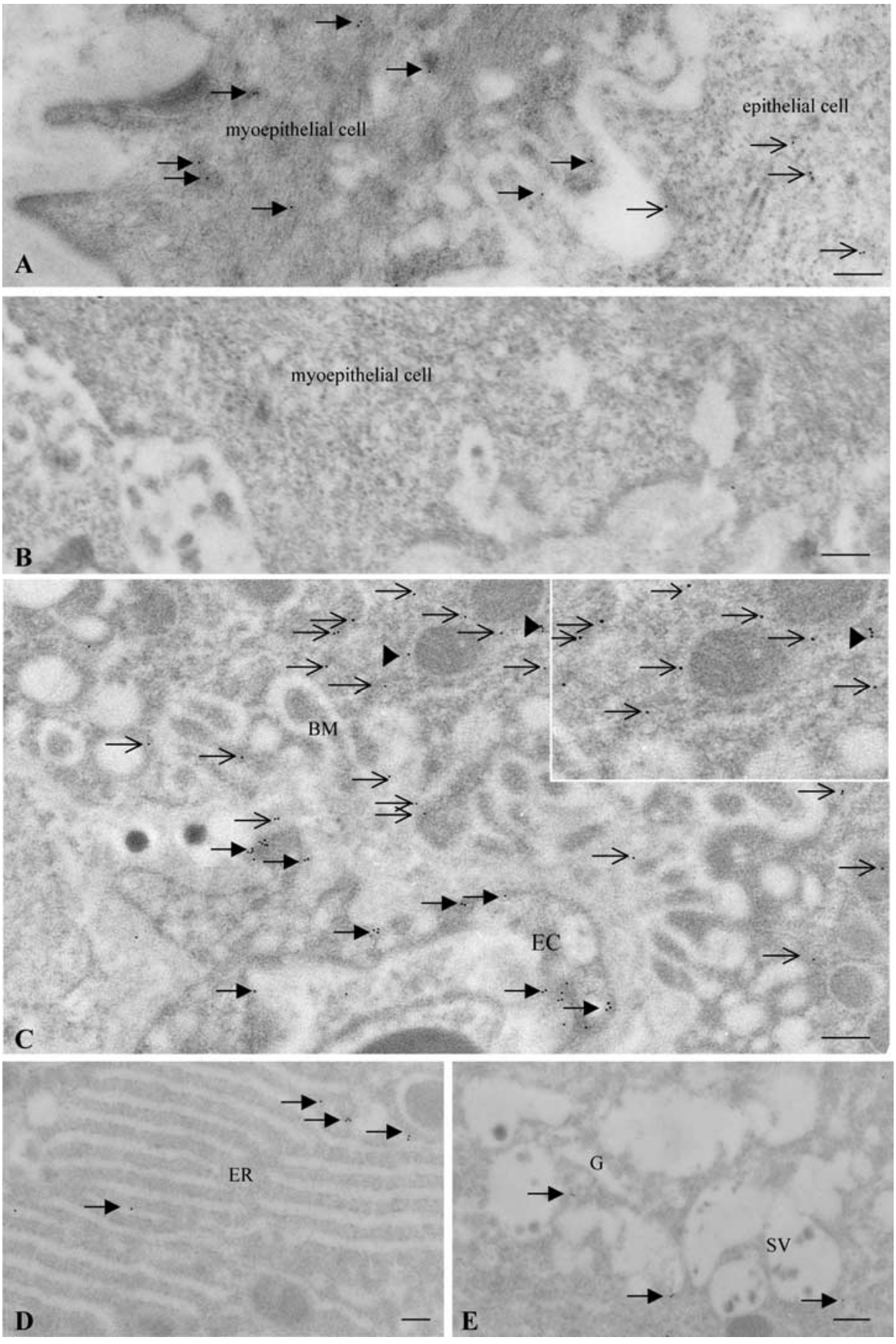


Figure 1. Immunofluorescence localization of oxytocin receptors in lactating rabbit and rat mammary acini
Mammary acini of 15 day lactating rabbits (*A*, *B*, *C* and *D*) and lactating rats (*E* and *F*) were fixed, permeabilized and treated for immunofluorescence without (*A*) or with a monoclonal oxytocin receptor antibody (2F8) followed by goat anti-mouse IgM-FITC (*B*, arrowheads). Actin was visualized with TRITC-phalloidin (*C* and *E*, arrows). Myoepithelial cells containing actin filling the cytoplasm were visualized (*C* and *E*, asterisk). Double localization of actin (arrows) and oxytocin receptors (arrowheads; *D* and *F*). Scale bar = 20 μm.



Oxytocin affects mammary tissue morphology

Since oxytocin binds to mammary epithelial cells, the question was to determine whether the hormone is able to have an effect on these cells. In order to investigate this point, the morphological features of the different mammary cell types were examined after 1–7 min incubation of lactating rabbit mammary fragments with or without 10^{-6} i.u. ml^{-1} of oxytocin.

Fragments incubated in control medium showed the typical morphological characteristics of acini. Elongated myoepithelial cells surrounded mammary epithelial cells, which contained abundant secretory components (secretory vesicles containing casein micelles and lipid droplets) and surrounded a lumen filled with milk components, casein micelles and lipid globules (Fig. 5*A* and *B*). At 1 min after the addition of oxytocin, the morphology of the myoepithelial cells and the size of the lumen were not strongly modified compared to the control. However, as revealed by photonic microscopy (Fig. 5*C*), milk constituents accumulated in the lumens of the acini and as revealed by electron microscopy, numerous secretory vesicles were located in the apical part of mammary epithelial cells or in close contact with the apical membrane (Fig. 5*D*). Seven minutes after addition of oxytocin, mammary epithelial cells appeared high and columnar (Fig. 5*F*). Myoepithelial cells were more contracted than the myoepithelial cells observed in fragments incubated in control medium and most of the acini lumens were small or completely collapsed (Fig. 5*E*). These morphological observations showed that addition of oxytocin *in vitro* to lactating rabbit mammary fragments induces an accumulation of milk constituents in the apical part of the mammary epithelial cells and in the acini lumens within 1 min. After 7 min, the morphological appearance of myoepithelial cells was characteristic of a contractile state. In order to support the apparent effect of oxytocin on secretory vesicle transport, quantification of the number of vesicles located in close contact with the apical membrane and in the 2.5 μm region closest to the apical membrane was performed (Table 1). After incubation for 1 min in the presence of 10^{-6} i.u. ml^{-1}, the number of vesicles in close contact with the apical membrane was more than 7-fold increased and the number of vesicles in the subapical region was about 2-fold increased. Despite these important increases, the results were not statistically significant probably due to individual variability. The total number of vesicles located in the apical region was 38% after incubation in Hanks' medium and 81% after incubation in the presence of oxytocin. The *P* value obtained by comparing the total number of vesicles in these two conditions was $P = 0.051$, very close to the 0.05 threshold. It showed that oxytocin addition increased the transport of vesicles to the apex of the epithelial cells (Table 1). These results suggest that after oxytocin

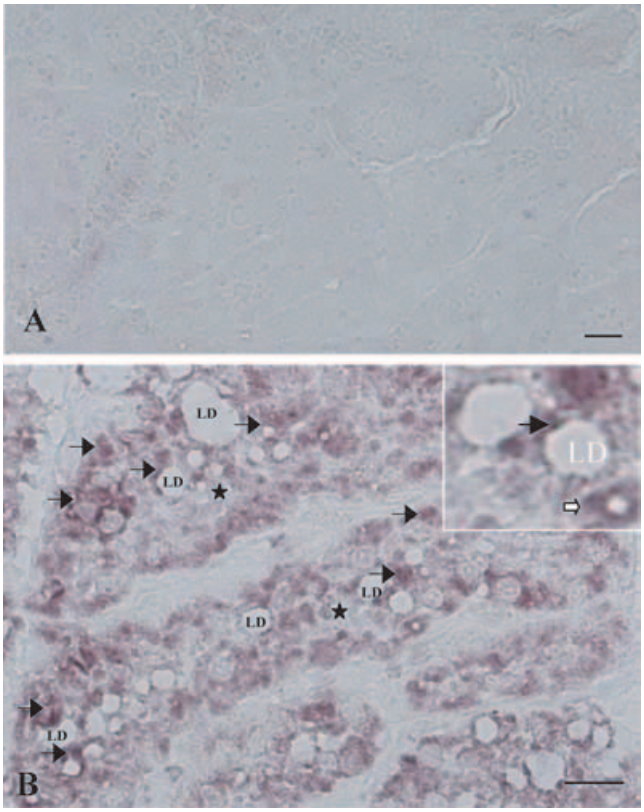


Figure 3. Detection of oxytocin receptor mRNA in lactating rat mammary gland by *in situ* hybridization analysis
Cryostat mammary sections of 15 day lactating rats were hybridized to a rat antisense oxytocin receptor-specific digoxigenin-labelled RNA (*B*) or to the corresponding sense RNA (*A*). Hybridization signals (oxytocin receptor mRNA) are visualized in the mammary epithelial cells, characterized by the presence of lipid droplets (*B*, arrows). LD, lipid droplets; ⋆, lumen; open arrow, myoepithelial cell. Scale bar = 20 μm.

Figure 2. Localization of oxytocin receptors in lactating rabbit mammary gland by immunoelectron microscopy
Ultrathin sections of 15 day lactating rabbit mammary gland were stained with (*A*, *C*, *D* and *E*) or without (*B*) a monoclonal oxytocin receptor antibody (2F8) followed by incubation with goat anti-mouse IgM antibody conjugated to 10 nm gold particles. Oxytocin receptors are visualized in myoepithelial cells (*A*, arrow) and in the endothelium of blood capillaries (*C*, arrow). Oxytocin receptors are also detectable in mammary epithelial cells, close to basal membrane (*A* and *C*, thin arrows), associated with electron-dense structures (*C*, arrowheads), on the endoplasmic reticulum (*D*, arrows) and in vesicles associated with the Golgi apparatus (*E*, arrows). BM, basal membrane of mammary epithelial cells; EC, endothelial cell; ER, endoplasmic reticulum; G, Golgi apparatus; SV, secretory vesicle. Scale bars in *A*, *B* and *C* = 100 nm, and in *D* and *E* = 200 nm.

addition to the incubation medium of the mammary fragments, a stimulating effect on the intracellular transport of the milk constituents in mammary epithelial cells might precede the contraction of myoepithelial cells.

Oxytocin affects mammary secretory process

To investigate more precisely the potential effect of oxytocin on the secretory process in mammary epithelial cells, intracellular localizations of α_{s1} casein, one of the major milk proteins, of annexin II, a protein interacting with membrane phospholipids and involved in exocytotic processes (Donnelly & Moss, 1997; Gerke & Moss, 1997), and of p58, a marker for the Golgi intermediate compartment (Saraste *et al.* 1987), were detected by immunofluorescence before and after addition of oxytocin.

In fragments incubated in control medium, immunocytochemically detectable α_{s1} caseins were mainly localized in the cytoplasmic apical region of mammary epithelial cells where secretory vesicles accumulated (Fig. 6*A*). Annexin II (Fig. 6*D*) and p58 (not shown) were localized in the supranuclear region. The myoepithelial cells stained by TRITC-phalloidin (Fig. 6*A* and *D*) and by specific labelling of smooth muscle actin (Fig. 6*G*) appeared as elongated cells at the basal part of the epithelium.

One minute after the addition of oxytocin, α_{s1} caseins accumulated near the apical membrane and in the lumens of acini, while the cytoplasm was not stained (Fig. 6*B*). Annexin II (Fig. 6*E*) and p58 (not shown) similarly accumulated in the apical part of the mammary epithelial cells. Most of the myoepithelial cells were elongated (Fig. 6*B*, *E* and *H*).

Seven minutes after the treatment, α_{s1} casein (Fig. 6*C*), annexin II (Fig. 6*F*) and p58 (not shown) were again detectable in the cytoplasmic supranuclear region of the mammary epithelial cells. A great number of myoepithelial cells appeared as constricted spots around the epithelium (Fig. 6*C*, *F* and *I*).

These results confirmed that after oxytocin addition to the incubation medium, transport of caseins and membrane markers precedes the contraction of myoepithelial cells.

In order to test the specificity of the oxytocin effect, an antagonist of oxytocin, atosiban, was added in the incubation medium before the addition of oxytocin. In our *in vitro* conditions, the addition of 10^{-8} g ml^{-1} of atosiban prevented both the oxytocin effect on the transport of the caseins (Fig. 7*Aa*) and the myoepithelial cell contraction (Fig. 7*Ab*). Electron microscopy showed that, whereas in the presence of oxytocin, numerous secretory vesicles were located close to the apex of the cell (Fig. 7*Ba*), in the presence of atosiban plus oxytocin for 1 min, secretory

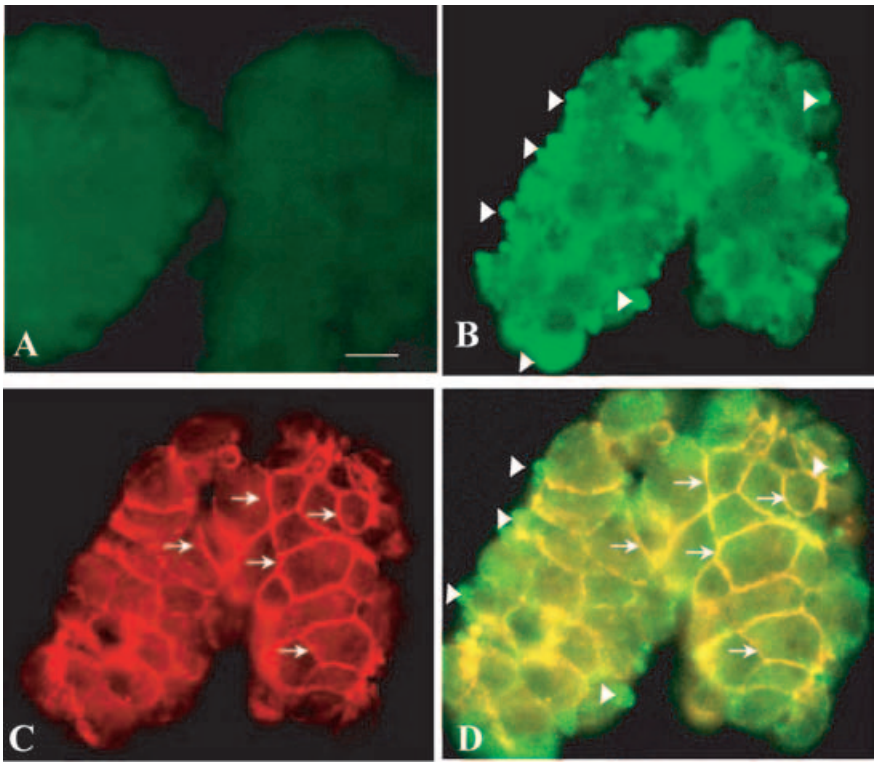


Figure 4. Localization of Fluo-Oxytocin on lactating rabbit mammary acini
A, mammary acini from 15 day lactating rabbits were permeabilized, incubated with 10^{-6} i.u. ml^{-1} oxytocin, fixed and then stained with Fluo-Oxytocin (50 nM). *B*, mammary acini were fixed and stained with Fluo-Oxytocin (50 nM; arrowheads). *C*, actin was visualized with TRITC-phalloidin (arrows). *D*, double localization of actin (arrows) and Fluo-Oxytocin (arrowheads). Scale bar = 20 μm.

vesicles were dispersed in the supranuclear region (Fig. 7*Bb* and Table 1).

Since oxytocin and vasopressin, two pituitary nonapeptide hormones, bind to the vasopressin receptor (Burbach *et al.* 1995) and atosiban binds to the vasopressin V1A receptor (Akerlund *et al.* 1999), it can be asked whether the effect of oxytocin on the secretory processes could be due to activation of the vasopressin receptor by oxytocin binding. To test this hypothesis, vasopressin was added to lactating rabbit mammary fragments. Vasopressin provoked a contraction of myoepithelial cells detectable 7 min following treatment (Fig. 8*C*), which is in line with the already known mimetic effect of oxytocin on myoepithelial contraction. Immunofluorescence localization of α_{s1} casein on lactating rabbit mammary tissue before and after addition of vasopressin for 1 (Fig. 8*B*) and 7 min (Fig. 8*C*) showed that, in contrast to what was observed after the addition of oxytocin, no modification of the localization of this protein was detectable. Similar results were observed with annexin II and p58 (not shown). In addition, no modification of secretory vesicle localization was observed in these mammary epithelial cells (Fig. 8*D* and Table 1). These results demonstrate that the effect of oxytocin on secretory processes is not a vasopressin-like effect.

Discussion

Results of the present investigation indicate for the first time that oxytocin receptors are expressed by lactating

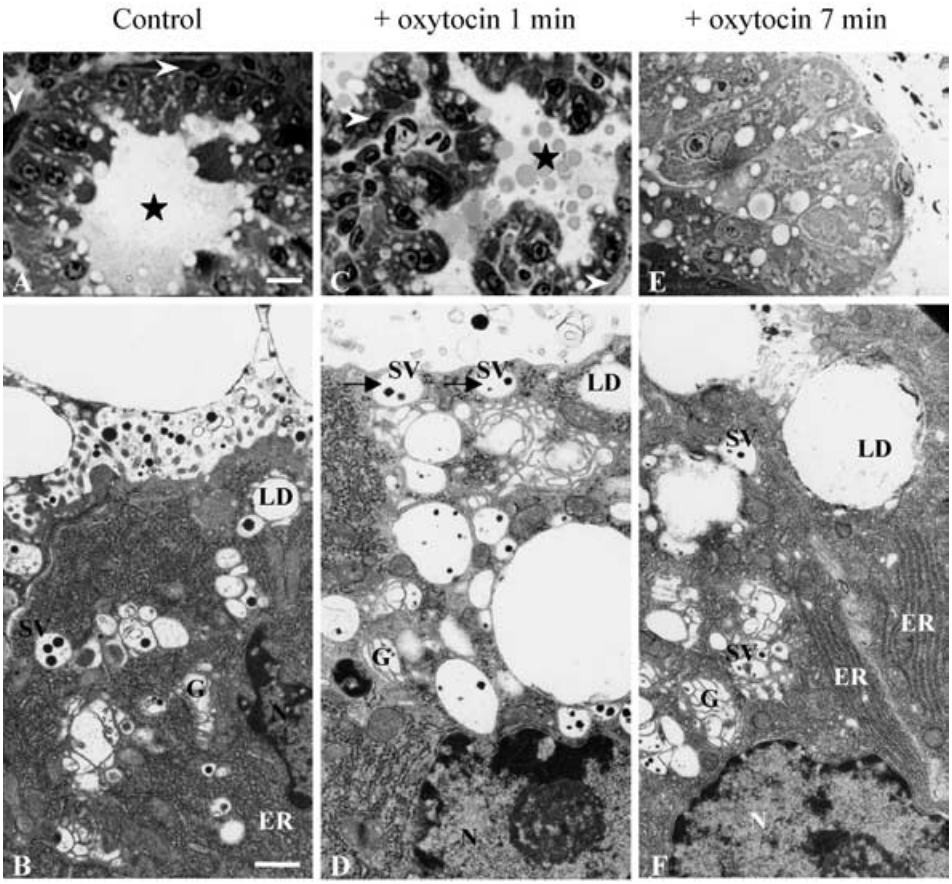


Figure 5. Morphological characteristics of lactating rabbit mammary explants incubated in the presence or absence of oxytocin

Mammary explants from a 15 day lactating rabbit were incubated in the absence (*A* and *B*) or presence for 1 min (*C* and *D*) and 7 min (*E* and *F*) of 10^{-6} i.u. ml^{-1} oxytocin, then fixed and treated for microscopy. Note that the lumen (⋆) was large in the absence (*A*) or presence (*C*) of oxytocin for 1 min and collapsed in the presence (*E*) of oxytocin for 7 min. In the absence of oxytocin, secretory vesicles were located in the cytoplasmic region between the nucleus and the apical membrane (*B*). In the presence of oxytocin for 1 min, numerous secretory vesicles were located in close contact with the apical membrane (*D*, arrows). Seven minutes after addition of oxytocin, the high and columnar mammary epithelial cells contained abundant endoplasmic reticulum (*F*). *A*, *C* and *E*, micrographs of 4 μm sections. Scale bar = 10 μm. *B*, *D* and *F*, electron microscopy micrographs. Scale bar = 1 μm. ER, endoplasmic reticulum; G, Golgi apparatus; LD, lipid droplets; ⋆, lumen; N, nucleus; SV, secretory vesicles. Myoepithelial cells are indicated by arrowheads.

Table 1. Number of secretory vesicles located in the apical region of mammary epithelial cells after 1 min incubation in the absence or presence of 10^{-6} i.u. ml^{-1} oxytocin, 10^{-6} i.u. ml^{-1} vasopressin, 10^{-8} g ml^{-1} atosiban and 10^{-8} g ml^{-1} atosiban + 10^{-6} i.u. ml^{-1} oxytocin

	No. of vesicles in close contact with apical membrane (%)	No. of vesicles in subapical region (%)	Total no. of vesicles in apical region (%)
Hanks' medium (3)	2.3 ± 0.3	36 ± 0.9	38 ± 1
Oxytocin (3)	15 ± 4	67 ± 2	81 ± 9*
Vasopressin (1)	1.4	35	36
Atosiban (1)	1.6	51	53
Atosiban + oxytocin (1)	5	44	49

Quantification was performed on electron micrographs. The number of vesicles in close contact with the apical membrane and the number of vesicles located in the region of 2.5 μm closest to the apical membrane were expressed as a percentage of the length (μm) of the corresponding apical membrane. In parentheses: number of animals. *P value, obtained by Student's t test to compare the increase in total number of secretory vesicles in apical region per micrometre of apical membrane of cells incubated in the presence of oxytocin *versus* cells incubated in Hanks' medium, is 0.051.

rabbit and rat mammary epithelial cells and that oxytocin is able to bind to these secretory cells. Moreover, a dual effect of oxytocin on the lactating mammary gland is shown, consisting of (1) within 1 min, an increase in the intracellular traffic of proteins and their release to the lumens of the acini, and (2) contraction of myoepithelial cells, detectable after 7 min.

The physiological effects of oxytocin are all mediated by one type of receptor. In the lactating mammary gland, oxytocin receptors are traditionally believed to be localized in myoepithelial cells (Soloff *et al.* 1980) to mediate contraction from the lumens of the alveoli to the ductules and ducts required for milk ejection (Ely & Petersen, 1941). Nevertheless, oxytocin receptors have been detected in ductal and/or glandular mammary epithelial cells from lactating and non-lactating humans and marmosets (Kimura *et al.* 1998). To determine whether oxytocin receptor localization in mammary epithelial cells could be generalized to different species, oxytocin receptor localization in the mammary epithelial cells of two species differing in their nursing pattern was studied: the rat, which spends about 18 h per day with young attached to her nipples and the rabbit, which has only one nursing period per day (Lincoln & Paisley, 1982).

The results obtained confirmed the presence of oxytocin receptors in myoepithelial cells. Moreover, oxytocin receptor synthesis was confirmed through the presence of oxytocin receptor mRNA in lactating rat mammary epithelial cells. In addition, the presence of oxytocin receptors in compartments of the biosynthesis pathway, such as RER and vesicles associated with the Golgi apparatus, is in agreement with its synthesis in mammary epithelial cells. Oxytocin receptors also appeared located close to the basal membrane, associated with unidentified small vesicles (coated pits, endosome-like structures and small tubulo-vesicular structures), which is a typical location after the binding of plasmatic oxytocin to its membrane receptors. These vesicles may be internalization sites of the hormone. Such an internalization of G-protein-coupled receptors in coated pits and vesicles has been shown in different cell types (Gaidarov *et al.* 1999). Moreover, the localization of oxytocin receptors in unidentified small tubulo-vesicular structures suggests that transport of this receptor might involve different pathways. New insights into internalization pathways have led to the idea that non-classical endocytic pathways, lipid raft dependent, associated or not with caveolin proteins, are involved in endocytosis (Le Roy & Wrana, 2005).

Oxytocin receptors are also detectable in endothelial cells of blood capillaries, allowing a mediation of the oxytocin effect on the control of vascular tone. Oxytocin receptors have already been detected in human vascular endothelial cells (Thibonnier *et al.* 1999) and in the rat great vessels (Jankowski *et al.* 2000).

In addition to the expression of oxytocin receptors by mammary epithelial cells, specific binding of oxytocin to rabbit mammary epithelial cells was observed. These results support the concept of lactating mammary epithelial cell responsiveness to oxytocin and then of the potential role of oxytocin on the mammary epithelium besides its effect on myoepithelial cells. Further evidence for a physiological role of oxytocin on mammary epithelial cells comes from previous localizations of oxytocin receptors in breast cancer-derived cell lines, mostly originating from epithelium (Taylor *et al.* 1990; Cassoni *et al.* 1994; Ito *et al.* 1996; Sapino *et al.* 1998),

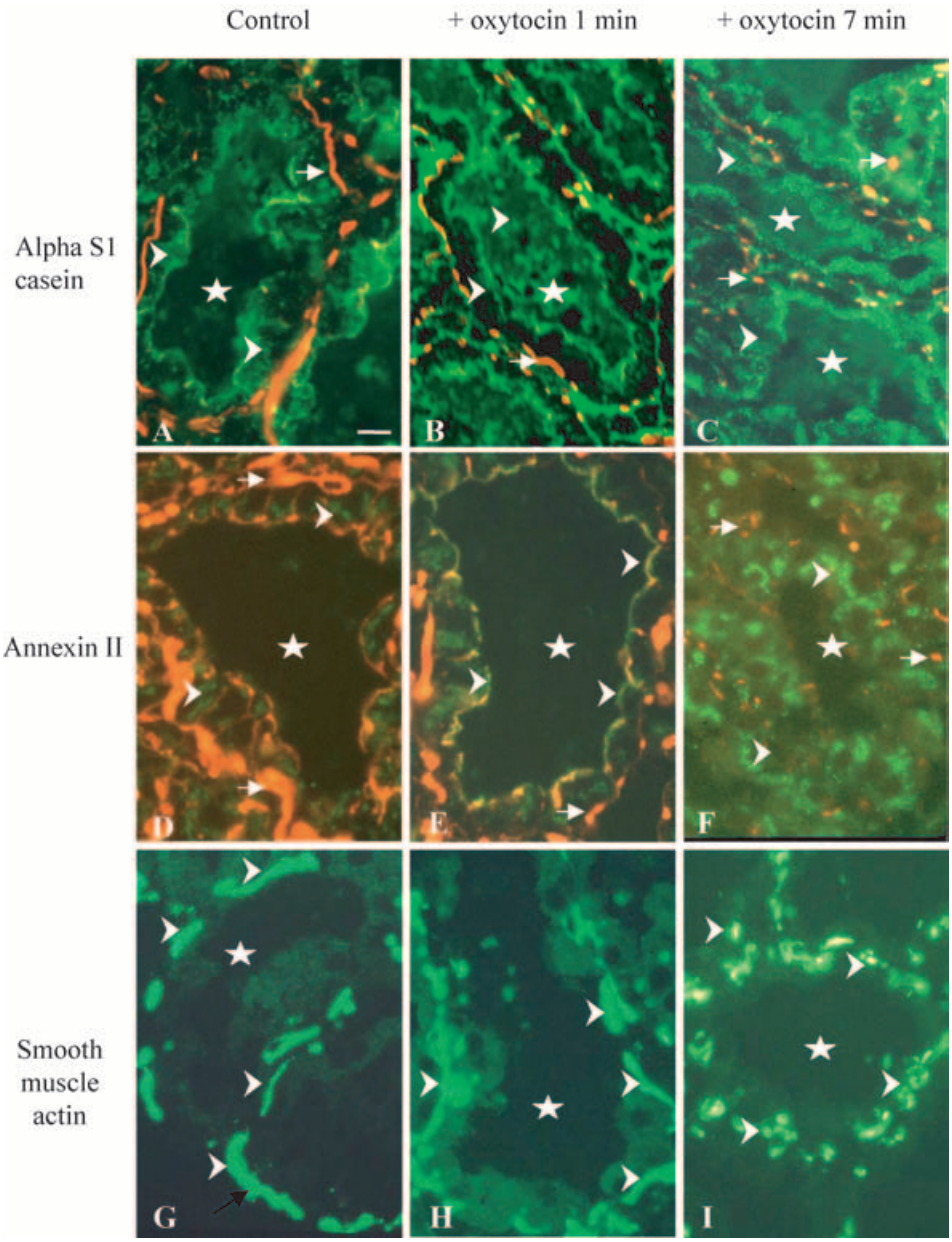


Figure 6. Immunofluorescence localization of α_{s1} casein and annexin II on lactating rabbit mammary tissue before and after addition of oxytocin and responsiveness of myoepithelial cells to oxytocin addition

Mammary explants from a 15 day lactating rabbit were incubated in the absence (*A*, *D* and *G*) or presence for 1 min (*B*, *E* and *H*) and 7 min (*C*, *F* and *I*) of 10^{-6} i.u. ml^{-1} of oxytocin, then fixed and treated for immunofluorescence. α_{s1} casein (*A*, *B* and *C*), annexin II (*D*, *E* and *F*) and smooth muscle actin (*G*, *H* and *I*) were visualized with specific antibodies and appropriate secondary FITC-antibodies (arrowheads). Actin was visualized with TRITC-phalloidin (*A*, *B*, *C*, *D*, *E* and *F*) (arrows). Note that in the absence of oxytocin, α_{s1} caseins were mainly localized in the cytoplasmic apical region of mammary epithelial cells (*A*) and annexin II (*D*) was localized in the supranuclear region. In the presence of oxytocin for 1 min, α_{s1} caseins accumulated near the apical membrane and in the lumens of acini (*B*), and annexin II (*E*) similarly accumulated in the apical part of the cells. In the presence of oxytocin for 7 min, α_{s1} casein (*C*) and annexin II (*F*) were detectable in the supranuclear region of the mammary epithelial cells. Note that myoepithelial cells appeared as elongated cells at the basal part of the epithelium in the absence of oxytocin (*G*) and in the presence of oxytocin for 1 min (*H*), while they appeared as constricted spots around the epithelium in the presence of oxytocin for 7 min (*I*). The lumen is indicated by a white star. Scale bar = 20 μm.

and from observations of *in vitro* effects of oxytocin on mammary cell proliferation. These effects vary from null (Ito *et al.* 1996) to anti-proliferative (Cassoni *et al.* 1994, 1996) or mitogenic (Taylor *et al.* 1990) according to culture conditions and cell line types.

In lactating ruminants, administration of exogenous oxytocin can increase milk production (Graf, 1969, 1970; Gorewit & Sagi, 1984; Nostrand *et al.* 1991; Ballou *et al.* 1993; Knight, 1994). For most authors, this increase in milk production associated with oxytocin injection can only be explained by a more efficient milk ejection (Knight, 1994) induced by the action of oxytocin on myoepithelial cell contraction. The galactopoietic effect of oxytocin could also result from the oxytocin effect on mammary epithelial cells. In this way, a double effect on lactating rabbit mammary tissue after *in vitro* addition of oxytocin was observed, consisting first of an acceleration of the intracellular transfer of secretory vesicles in mammary epithelial cells and secondly in a contraction of myoepithelial cells. This result confirms previous work by Ollivier-Bousquet (1976), which demonstrated that oxytocin stimulates *in vitro* intracellular transport of newly synthesized caseins from RER to the Golgi apparatus and secretory vesicles in lactating rabbit mammary fragments.

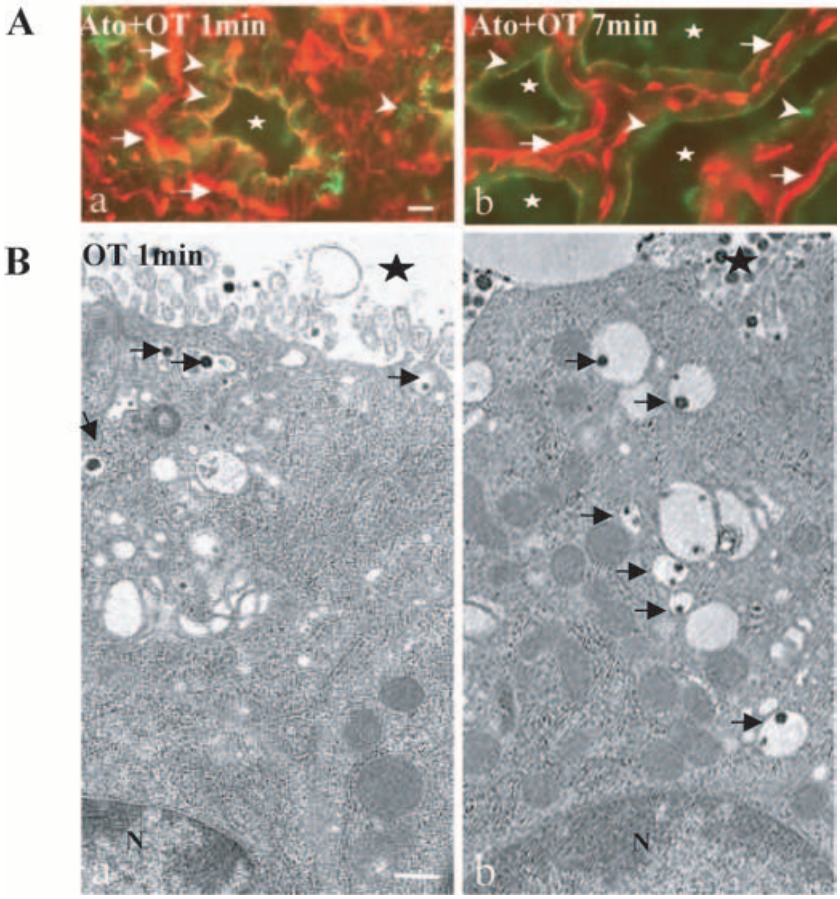


Figure 7. Immunofluorescence localization of α_{s1} casein and morphological characteristics of lactating rabbit mammary explants after incubation in the presence of oxytocin and in the presence of atosiban and oxytocin

A, mammary explants from a 15 day lactating rabbit were incubated in the presence of 10^{-8} g ml^{-1} atosiban (Ato) for 3 min and in the presence of 10^{-6} i.u. ml^{-1} oxytocin (OT) for 1 min (*Aa*) and 7 min (*Ab*), then fixed and treated for immunofluorescence. α_{s1} casein was visualized with specific antibody and appropriate secondary FITC-antibody (arrowheads). Actin was visualized with TRITC-phalloidin (arrows). Note that in the presence of atosiban and oxytocin for 1 min, caseins are present in the supranuclear cytoplasmic region (*Aa*). In the presence of atosiban and oxytocin for 7 min, myoepithelial cells were still elongated (*Ab*). The lumen is indicated by a white star. Scale bar = 10 μm. *B*, morphological features of 15 day lactating rabbit mammary explants incubated in the presence of 10^{-6} i.u. ml^{-1} oxytocin for 1 min (*Ba*) or in the presence of 10^{-8} g ml^{-1} atosiban for 3 min and the presence of 10^{-6} i.u. ml^{-1} oxytocin for 1 min (*Bb*). In the presence of oxytocin, numerous secretory vesicles were located close to the apex of the cell (*Ba*), whereas in the presence of atosiban plus oxytocin for 1 min, secretory vesicles were dispersed in the supranuclear region (*Bb*). ★, lumen; N, nucleus; secretory vesicles are indicated by arrows. Scale bar = 1 μm.

The emptying of the mammary epithelial cells might avoid a negative feedback of accumulation of the milk constituents and/or may stimulate synthesis of new milk protein.

This effect on both epithelial and myoepithelial cells is a specific oxytocin effect since addition of atosiban, a competitive oxytocin receptor antagonist (Melin *et al.* 1986), in our conditions, was able to inhibit both the effects of oxytocin. However, addition of high concentrations of atosiban has had a different and mimetic effect (authors' unpublished results). Atosiban has been described as a 'biased agonist' of the oxytocin receptors in human myometrial cells (Reversi *et al.* 2005). The effect of atosiban at pharmacological concentrations on mammary epithelial cells remains to be clarified.

Oxytocin and vasopressin are structurally related nonapeptide hormones that differ from each other only by two amino acids. They serve separate hormonal functions in the periphery via different receptor subtypes but they both show relatively high affinity for all receptor subtypes (Burbach *et al.* 1995). The stimulation of inositol phosphate formation in rat mammary gland by oxytocin

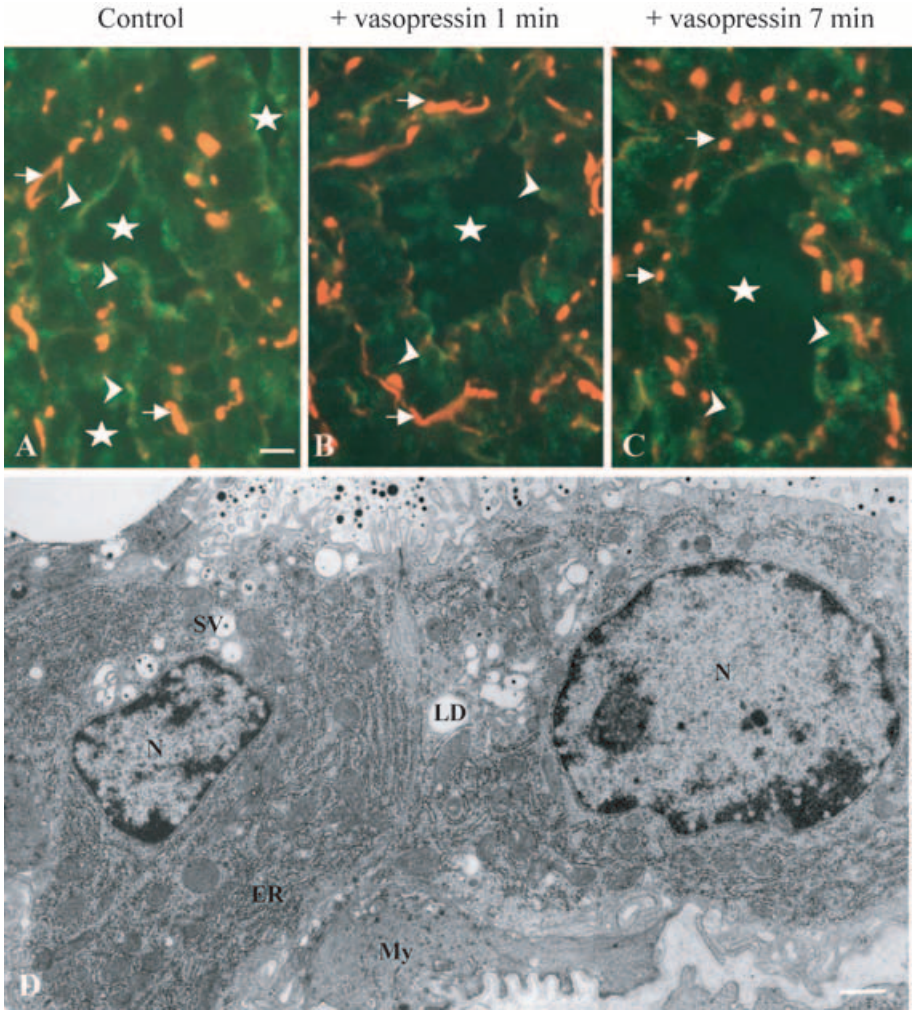


Figure 8. Immunofluorescence localization of alpha-S1 casein and morphological features of lactating rabbit mammary explants after incubation in the presence of vasopressin
Mammary explants from a 15 day lactating rabbit were incubated in the absence (*A*) or presence for 1 min (*B*) and 7 min (*C*) of 10^{-6} i.u. ml^{-1} of vasopressin, then fixed and treated for immunofluorescence. α_{S1} casein was visualized with specific antibody and appropriate secondary FITC-antibody (arrowheads). Actin was visualized with TRITC-phalloidin (arrows). Note that addition of vasopressin for 1 min (*B*) and 7 min (*C*) provoked no modification of the localization of the α_{S1} casein. In the presence of vasopressin for 7 min, myoepithelial cells were contracted (*C*). The lumen is indicated by a white star. Scale bar = 20 μm. *D*, morphological features of 15 day lactating rabbit mammary explants incubated in the presence of 10^{-6} i.u. ml^{-1} of vasopressin for 1 min. No modification of secretory vesicle localization was observed in these cells. ER, endoplasmic reticulum; LD, lipid droplets; My, myoepithelial cell; N, nucleus; SV, secretory vesicle. Scale bar = 1 μm.

occurs through the occupancy of vasopressin receptor sites (Soloff *et al.* 1989). Furthermore V1A receptors and oxytocin receptors may be coexpressed in the same tissue, as described in myometrium (Tence *et al.* 1990). Atosiban also binds to the vasopressin V1A receptor (Akerlund *et al.* 1999). Moreover, it has been suggested that vasopressin could play a role in mammary gland function similar to the action of oxytocin. For example, vasopressin increases milk flow and milk fat concentration in the lactating goat probably by action on myoepithelium (Olsson *et al.* 2003) and acts as a stimulator of tumour cell growth (Taylor *et al.* 1990). So, the question arises as to whether the present observations on mammary epithelial cells resulted from a vasopressin-like effect. Results of *in vitro* addition of vasopressin to lactating mammary explants confirm the ability of vasopressin to provoke milk ejection by contraction of myoepithelial cells without a direct effect on intracellular transport of milk constituents in mammary epithelial cells. Consequently, the secretory effect of oxytocin on mammary epithelial cells may be considered as a specific oxytocin effect.

Accordingly, the present results are consistent not only with the classical role of oxytocin in myoepithelial contraction and alveolar emptying, but also with a direct effect on mammary secretory epithelium. This raises the question of how oxytocin can have different effects depending on the cell type. One possibility is that in the mammary tissue different effects are mediated by different oxytocin receptor subtypes, since two oxytocin receptor mRNA species, but encoding the same oxytocin receptor, have been described in the mammary gland (Breton *et al.* 2001). One mRNA was found to be common to all oxytocin-responsive tissues whereas the other mRNA was unique to the mammary gland, suggesting a differential control of oxytocin receptor expression according to tissue type. Since a differential control exists according to tissue type, such control might exist in the mammary gland according to cell types (myoepithelial *versus* epithelial cells). Another way for oxytocin to assume several functions may be heterodimerization of the oxytocin receptor with another G-coupled protein receptor such as the vasopressin V1A receptor, as described in transfected human embryonic kidney cells (Terrillon *et al.* 2003). Oxytocin binding to either heterodimers or native forms of oxytocin receptor may have functional implications. Finally, the signal transduction involved in the biological effect of oxytocin on breast cancer is different depending on whether oxytocin promotes or inhibits proliferation (Cassoni *et al.* 2001). Why different pathways depending on target cells could not exist?

In conclusion, the present results are consistent with a putative role for oxytocin in the regulation of the secretory activity of the lactating mammary gland in addition to its myoepithelial cell contraction effect. The precise mechanism by which oxytocin stimulates the secretion of milk components from mammary epithelial cells clearly requires further study. Moreover, oxytocin receptor location in small vesicles close to the basal membrane was observed. It might be interesting to characterize these vesicles more precisely. This could throw new light on oxytocin receptor trafficking. In addition, the steps of intracellular signalling cascades subsequent to the binding of oxytocin to its receptor on secretory mammary epithelial cells remain unknown and it would be useful to study these events and their connection with biological effects.

In the *in vitro* studies, comparable results were obtained in the rabbit and rat. It is therefore important to be conscious that in these two species without milk storage compartments, the natural pattern of nursing and then milk ejection vary from once a day in the rabbit to several times a day in the rat. Consequently, the different patterns of oxytocin release should modulate the importance of oxytocin-induced mammary epithelial cell emptying according to species. Thus, it may be absolutely necessary for the rabbit to empty its whole mammary gland (mammary epithelial cells and alveoli lumens) during its only daily nursing, whereas this phenomenon would be less important in the rat.

In the light of these results, the classical and unique role of oxytocin on milk ejection needs to be re-evaluated by taking its additional effect on secretory processes into consideration.

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Supplemental material

The online version of this paper can be accessed at:
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and contains supplemental material consisting of three figures entitled:

Figure S1. Morphological aspect of acini from lactating rabbit mammary fragments incubated in the presence (A, C) or absence (B) of 10^{-8} g/L of atosiban and in the presence (B, C) or absence (A) of 10^{-6} IU/mL of oxytocin for 1 minute.

Figure S2. Morphological aspect of acini from lactating rabbit mammary fragments incubated in the presence (A, C) or absence (B) of 10^{-8} g/L atosiban and in the presence (B, C) or absence (A) of 10^{-6} IU/mL of oxytocin for 7 minutes.

Figure S3. Morphological aspect of myoepithelial cells from lactating rabbit mammary fragments incubated in the presence or absence of 10^{-6} IU/mL of oxytocin.

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